



Nutrients utilization, methane emission, immune function, blood metabolites and performance of buffalo calves fed *Trachyspermum copticum* seed oil

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Received: 7 April 2017; Accepted: 1 August 2018

ABSTRACT

The effect of ajwain seed oil (*Trachyspermum copticum*, AjO) on nutrient digestibility, methane emission, immune status, blood metabolites and growth performance was studied on 15 growing male buffalo calves. The animals were divided into three groups in completely randomized design and assigned to three dietary treatments, viz. control without additive (T₁), AjO at the rate of 1 ml/calf/day (T₂) and AjO at the rate of 2 ml/calf/day (T₃). Feeding was continued for 120 days. The dry matter intake (kg/d) was higher by 3 and 8% and average daily weight gain by 10 and 16% in T₂ and T₃ groups as compared to control (T₁) group, but differences were not significant. There was no effect on apparent digestibility of dry matter, organic matter, ether extract, neutral detergent fibre and acid detergent fibre except crude protein digestibility which was higher in T₃ group as compared to control. The methane production and energy metabolism were not changed by feeding of AjO. The animals of T₃ group were in higher nitrogen balance accompanied with low blood urea level. The blood metabolites and immune status (cell mediated and humoral immune response) reflecting health of the animals, were similar and within normal range in all the groups. Though AjO feeding could not affect the overall performance of the animals but was able to modulate protein metabolism resulting in improvement in protein utilization efficiency.

Key words: Buffalo calves, Essential oil, Feed additive, Growth, Methane

Many natural compounds used as alternative to antibiotics in animal feeding have been shown to express positive effects on growth performance and different health parameters (Windisch *et al.* 2008, Vohra *et al.* 2016, He *et al.* 2017) and therefore, can be good replacers of antibiotics. One such alternative is the use of essential oils (EOs), which are referred as volatile or ethereal oils, acquired from plant materials that are extracted by steam and/or water distillation. Chemically, EOs are a blend of secondary metabolites commonly composed of terpenoids and phenylpropanoids, which are generally recognized as safe by the Food and Drug Administration (FDA 2004). The EOs from various sources individually or in combination have been studied as rumen modifiers (Pawar *et al.* 2014, Cobellis *et al.* 2016) and as an anti-methanogen in *in vitro* system (Kamra *et al.* 2006, Kumar *et al.* 2009). Based on the chemical composition and the results of previous *in vitro* experiments, one essential oil (*Trachyspermum copticum*, ajwain seed oil) exhibiting antimethanogenic activity, was selected for feeding trials on buffalo calves. The seeds (source of oil) of this plant has anti-microbial activity

(Sivropoulou *et al.* 1996) due to the presence of various phytochemical constituents mainly glycosides, saponins, phenolic compounds, volatile oils (thymol, γ -terpinene, para-cymene, and α - and β -pinene, containing about 50% thymol) (Bairwa *et al.* 2012). Therefore, the present study was conducted to examine the effect of feeding ajwain seed oil (*Trachyspermum copticum*, AjO) on growth performance, nutrient utilization, methane emission, immune functions and blood metabolites of buffalo calves.

MATERIALS AND METHODS

Animals and experimental design: Male buffalo (*Bubalus bubalis*) calves (15), 8–10 months old, were divided into three groups in completely randomized design and subjected to three dietary treatments, viz. control with no additive (T₁), supplemented with AjO at the rate of 1 ml/calf/day (T₂) and supplemented with AjO at the rate of 2 ml/calf/day (T₃). AjO was mixed thoroughly in the concentrate mixture and complete consumption of this concentrate mixture was ensured after that wheat straw was offered. During the experimental period, all the calves were kept in a well-ventilated shed with individual feeding and watering arrangement. The animals were fed concentrate mixture (composed of crushed maize grain, 32; wheat bran, 45; deoiled soybean meal, 20; mineral mixture, 2 and common salt, 1 part) and wheat straw in 1:1 ratio to meet their nutrient requirement as per ICAR (1998) for 120 days. The body

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weight changes were recorded fortnightly. The experimental protocol followed in this experiment was approved by the Institutional Animal Ethics Committee.

Apparent nutrient digestibility and methane emission study: A metabolism trial of 6 days collection was conducted after 45 d of experimental feeding. The animals were shifted in metabolic cages and collection was started after 2 days of acclimatization period. Daily feed offered, residue, feces and urine were collected individually. After measuring the total amount, representative samples of residual feed, feces and urine were preserved for analysis. After metabolism trial, methane emission was measured in an open circuit respiration chamber. The chamber was maintained at 25°C and 65% relative humidity with air flow rate of about 250 L/min. Methane was measured in air going in and coming out of the chamber through an infrared gas analyzer (Model 300, Analytical Development Co. Ltd., Hoddesdon, England). Gross energy (GE) of feed, faeces and urine samples was estimated by using Gallenkamp ballistic bomb calorimeter according to the manufacturer's manual.

Chemical analyses: The samples of feed offered, residual feed and faeces were analyzed for dry matter (DM), organic matter (OM) and ash, crude protein (CP) and ether extract (EE) as per AOAC (1995). Neutral detergent fibre (NDF; estimated without amylase and sodium sulphite and expressed inclusive of the ash content), acid detergent fibre (ADF; also inclusive of ash content) and acid detergent lignin were analyzed as per Van Soest *et al.* (1991). Nitrogen balance was calculated by estimating nitrogen in feed offered, residual feed, faeces and urine.

Immune response: At 90 days of experimental feeding, all calves were sensitized subcutaneously in the left mid-cervical region with 1 mg ovalbumin (Bangalore Genei, India) dissolved in 1 ml of sterile phosphate buffer saline (PBS), diluted 1:1 (vol/vol) in incomplete Freund's adjuvant. A subsequent injection of 1 mg of ovalbumin in sterile PBS without adjuvant was administered 14 d later as a booster. The blood samples were collected at 0, 14 and 21 days post-vaccination to determine anti-ovalbumin antibody titer using indirect ELISA as described elsewhere (Coligan *et al.* 1991). The cell-mediated immune (CMI) response was assessed at the end of experimental feeding by measuring increase in skin thickness as delayed-type hypersensitivity (DTH) reaction. The skin on both sides of the neck was cleaned and shaved with a razor 24 h prior to injection, so that any inflammation set during shaving or due to abrasion could subside. Then 150 µg of phytohaemagglutinin-p (PHA-P) in 200 µl of PBS (pH 7.4) was injected intra-dermal at two different sites 4 cm apart. The difference in skin fold thickness in millimeter (mm) between PHA-P injected and control sites was measured with the help of Vernier caliper at 0, 24, 48, 72 and 96 h post sensitization.

Blood metabolites: The blood samples from jugular vein were collected from each calf in the vials with and without EDTA. The haemoglobin (Hb) in blood was determined by cyanmethemoglobin method (Dacie and Lewis 1975) and

packed cell volume (PCV) was determined by capillary method. The serum samples were analyzed for glucose, total proteins, albumin, globulin, lactate dehydrogenase (LDH), serum glutamate pyruvate transaminases (SGPT), serum glutamate oxalate transaminases (SGOT), triglycerides, cholesterol and urea concentrations using diagnostic kits (Span Diagnostics Ltd., Surat, India).

Statistical analyses: All the data were statistically analyzed according to a completely randomized design using the general linear model (GLM) procedure of SPSS version 12.0 (SPSS Inc., Chicago IL). Significant differences between means of treatments were assessed by the Duncan's test and the differences among treatments were declared significant at $P < 0.05$.

RESULTS AND DISCUSSION

Growth performance: Feeding of AJO to the buffalo calves, showed a trend of increased daily dry matter intake (DMI) (kg/d) as there was 3 and 8% higher DMI in T₂ and T₃ groups as compared to control. The average daily weight gain (ADG) was 10 and 16% higher in the animals of T₂ and T₃ groups as compared to that of control (Table 2), resulting in 5.6% higher feed conversion efficiency (FCR) in both the treated groups again depicting a improving trend in growth performance of buffalo calves by feeding AJO. Yang *et al.* (2010) observed improved growth performance in steers by dietary supplementation of cinnamaldehyde at 400, 800, or 1,600 mg/steer/d and Benchaar *et al.* (2006) reported that EO mixture (consisting of thymol, eugenol, vanillin and limonene) had a quadratic effect on feed conversion efficiency. The FCR was improved at the dose of 2 g/d but not at the dose of 4 g/d in beef cattle fed silage based diet; however, ADG was not affected with either of the doses. However, Beauchemin and McGinn (2006) observed no change in ADG of cattle supplemented with a mixture of essential oils (Crina Ruminants; 1g/d). The discrepancies in the findings of various experiments might be due to differences in the EOs, their doses, animal, physiological state of life, diet etc. Soltan *et al.* (2009) reported detrimental effect of EOM (eucalyptus oil, menthol crystal, mint oil) feeding at higher doses to Holstein male calves during both pre-weaning and post-weaning periods,

Table 1. Chemical composition (% DM basis) of concentrate mixture and wheat straw

Chemical composition	Concentrate mixture	Wheat straw
Organic matter	92.37	92.72
Crude protein	19.67	3.04
Ether extract	3.89	1.36
Neutral detergent fibre	37.73	75.92
Acid detergent fibre	18.32	47.29
Hemicellulose	19.42	28.63
Cellulose	14.43	36.61
Acid detergent lignin	3.89	10.68
Total ash	7.63	7.28
Acid insoluble ash	2.85	4.31

Table 2. Effect of feeding *Trachyspermum copticum* seed oil (AjO) on body weight changes and feed conversion ratio in buffalo calves

Parameter	T ₁	T ₂	T ₃	SEM	P value
Initial BW (kg)	71.16	70.80	71.88	10.18	0.994
Final BW (kg)	107.8	112.0	116.0	12.20	0.713
Net BW gain (kg)	36.68	40.42	43.11	3.645	0.141
Average daily gain (g)	311.3	342.6	361.8	29.92	0.273
Total DM intake (kg)	322.7	332.3	348.3	39.60	0.843
Feed conversion ratio	8.73	8.24	8.24	0.646	0.692

T₁, control; T₂, AjO @ 1 ml/calf/day; T₃, AjO @ 2 ml/calf/day; BW, body weight; DM, dry matter.

whereas lower doses were beneficial for the animals. The EOs are more effective in their active state that is undissociated hydrophobic form which depends on the pH, which again depends on diet composition, hence EOs response is directed by the type of diet (Cardozo *et al.* 2005) as well as the type of essential oil used as a rumen modifier.

Apparent nutrient digestibility: There was no difference (P>0.05) in intake and apparent digestibility of DM, OM, EE, NDF and ADF in the three groups (Table 3), however, CP digestibility was significantly (P<0.05) higher in T₃ group but it was comparable with T₂. The intakes of DM, OM, NDF and ADF by dietary supplementation of garlic oil or juniper berry oil (2 g/cow/d) were not affected in cows (Yang *et al.* 2007).

Table 3. Effect of feeding *Trachyspermum copticum* seed oil (AjO) on intake and apparent digestibility of nutrients in buffalo calves

Parameter	T ₁	T ₂	T ₃	SEM	P value
Nutrient intake (kg/d)					
Dry matter	2.50	2.35	2.86	0.437	0.506
Organic matter	2.33	2.19	2.67	0.409	0.507
Crude protein	0.319	0.311	0.368	0.050	0.485
Ether extract	7.22	6.95	8.31	1.159	0.491
NDF	1.46	1.33	1.66	0.274	0.511
ADF	0.76	0.69	0.86	0.145	0.523
Digestibility (%)					
Dry matter	62.54	61.32	64.26	2.001	0.377
Organic matter	64.75	63.46	66.22	1.969	0.413
Crude protein	61.68 ^a	64.05 ^{ab}	66.36 ^b	1.216	0.013
Ether extract	74.67	73.26	74.52	2.148	0.778
NDF	56.38	55.09	59.50	2.720	0.298
ADF	45.80	44.94	47.13	2.100	0.593

^{ab}Means with different superscript in a row differ significantly. T₁, control; T₂, AjO @ 1 ml/calf/day; T₃, AjO @ 2 ml/calf/day; NDF, neutral detergent fibre; ADF, acid detergent fibre.

Nitrogen balance: In present study, nitrogen balance (Table 4) was significantly (P<0.05) higher in T₃ group. Patra *et al.* (2011) also reported higher nitrogen balance in sheep fed on garlic supplemented diet as compared to control. Yang *et al.* (2007) observed increased ruminal digestibility of nitrogen by dietary supplementation of garlic oil (5 g/cow/d). In contrast, the retention of N was not

Table 4. Effect of feeding *Trachyspermum copticum* seed oil (AjO) on nitrogen intake and balance during metabolism trial of buffalo calves

Parameter	T ₁	T ₂	T ₃	SEM	P value
N intake (g/d)	51.14	49.75	59.02	7.977	0.485
Faecal N loss (g/d)	19.58	17.93	19.90	3.202	0.808
Urine N loss (g/d)	19.39	17.92	23.61	4.175	0.405
N balance (g/d)	12.17 ^a	13.91 ^{ab}	15.52 ^b	1.107	0.043

^{ab}Means with different superscript in a row differ significantly. T₁, control; T₂, AjO @ 1 ml/calf/day; T₃, AjO @ 2 ml/calf/day;

affected in lactating dairy cows (Benchaar *et al.* 2006) or in beef cattle (Benchaar *et al.* 2006) fed different doses (2 and 4 g/head/d) of a mixture of essential oil compounds.

Methane emission and energy metabolism: Methane production in terms of l/kg DMI and l/kg DDMI was reduced by 5.4 and 3.9% in T₂ group and 5.8 and 8.5% in T₃ group (Table 5). There was no impact of AjO feeding on energy metabolism at both the doses (Table 5). Wang *et al.* (2009) observed lower methane emission by inclusion of 0.25 g/day of EO mixture from oregano plants in the diet of sheep for 15 d. Patra *et al.* (2011) also did not found any effect of garlic (rich in EOs) feeding on methane production in sheep. No effect on methane production by feeding of EOs mixture (1 g/d) for 21 days in beef cattle was also reported by Beauchemin and McGinn (2006). Tomkins *et al.* (2015) could not obtained reduction in methane production by feeding a blend of EOs (CRINA®

Table 5. Effect of feeding *Trachyspermum copticum* seed oil (AjO) on methane emission and energy metabolism of buffalo calves

Parameter	T ₁	T ₂	T ₃	SEM	P value
Body weight (kg)	93.58	91.30	100.7	4.246	0.686
DM intake (kg/d)	2.26	2.12	2.78	0.391	0.261
Methane emission					
CH ₄ (l/d)	38.50	34.00	45.75	4.965	0.128
CH ₄ (l/kg DMI)	17.57	16.63	16.56	1.169	0.641
CH ₄ (l/kg DDMI)	28.16	27.06	25.78	1.620	0.379
Energy metabolism					
GE intake (Mcal/d)	11.06	10.47	13.48	1.835	0.352
Faecal GE loss (Mcal/d)	4.73	4.31	5.39	0.773	0.259
GE digestibility (%)	57.24	58.75	59.98	2.676	0.396
Urine GE loss (Mcal/d)	0.20	0.19	0.24	0.097	0.593
CH ₄ GE loss (Mcal/d)	0.37	0.33	0.43	0.046	0.691
% GE intake loss as CH ₄	3.37	3.17	3.22	0.212	0.326
ME (%)	51.98	53.84	55.11	2.912	0.251

^{ab}Means with different superscript in a row differ significantly. T₁, control; T₂, AjO @ 1 ml/calf/day; T₃, AjO @ 2 ml/calf/day; DM, dry matter; CH₄, methane; GE, gross energy; ME, metabolizable energy.

Table 6. Effect of feeding *Trachyspermum copticum* seed oil (AjO) on cell mediated immunity assessed by DTH response (increase in skin thickness, mm)

Hours Post-inoculation	Treatment			Mean	SEM	P value		
	T ₁	T ₂	T ₃			T	P	T*P
0	5.55	5.63	5.53	5.57 ^a	0.166	0.739	0.001	0.977
24	9.10	9.08	9.18	9.12 ^e				
48	8.13	8.23	8.08	8.14 ^d				
72	7.53	7.45	7.40	7.46 ^c				
96	6.18	6.30	6.38	6.28 ^b				
Mean	7.30	7.34	7.31					

^{abcde}Means with different superscript in a column differ significantly. ^{ab}Means with different superscript in a row differ significantly. T₁, control; T₂, AjO @ 1 ml/calf/day; T₃, AjO @ 2 ml/calf/day.

Table 7. Effect of feeding *Trachyspermum copticum* seed oil (AjO) on humoral immune response

Treatment	Anti-ovalbumin titers (OD ₄₅₀)	
	Primary response	Secondary response
T ₁	0.465	0.696
T ₂	0.477	0.715
T ₃	0.469	0.643
SEM	0.098	0.111
P value	0.352	0.295

T₁, control; T₂, AjO @ 1 ml/calf/day; T₃, AjO @ 2 ml/calf/day.

Ruminants) to beef cattle. It has been observed that EOs has been worked out majorly in *in vitro* system barring few feeding trials. Most of the *in vitro* experiments showed reduction in methane production but similar response could not be obtained in feeding trials. Apparently, the main reason might be that in *in vitro* system one can try as many combinations as possible but in animal experimentation it is not possible.

Immune response: The cell mediated immune response (cytotoxic T lymphocytes, natural killer cells and macrophages) is responsible for getting rid of intracellular pathogens, virus infected cells and tumor cells (Kuby 1994). When PHA-P or any other antigen is injected into the body, the cell mediated immune system responds to destroy the foreign substances. In present study, supplementation of EO did not have any significant effect on the CMI as assessed DTH response (Table 6). Also, the humoral immune response against ovalbumin (titer at OD₄₅₀) did not differ (P>0.05) among the treatments, either as primary or secondary response (Table 7). It was speculated that addition of EO in the diet may influence immune response as they have antimicrobial activity (Elgayyar *et al.* 2001). However, results of present study showed that supplementation of AjO had no effect on immune response either cell mediated or humoral in buffalo calves.

Blood metabolites: There were no differences (P>0.05) in the concentrations of blood haemoglobin and packed cell volume among the treatments (Table 8). The serum concentration of glucose, total proteins, albumin, globulin,

triglycerides, cholesterol, LDH, SGPT and SGOT were not affected by AjO supplementation. In this study, serum urea concentration was significantly (P<0.05) decreased in AjO supplemented groups as compared to control. Supplementation of diets with EO is thought to decrease ruminal proteolysis by inhibiting the conversion of amino acids to ammonia (Calsamiglia *et al.* 2007, Benchaar *et al.* 2008). In accordance to previous researches (Yang *et al.* 2010), EO supplementation did not significantly affect most of the blood metabolite concentrations. However, it has been reported that concentrations of some blood metabolites such as triglycerides can be influenced by EO supplementation by change in feed intake (Yang *et al.* 2010) and urea concentration may be altered depending upon ruminal ammonia-N production enhancement or inhibition as it is absorbed across the rumen wall into portal blood, and most of it is converted to urea in the liver. Therefore, synthesis of urea in the liver depends on the amount of ammonia absorbed from the rumen; as a result, urea N concentration in blood is highly correlated with the rumen NH₃-N

Table 8. Effect of feeding *Trachyspermum copticum* seed oil (AjO) on blood metabolites of buffalo calves

Parameter	T ₁	T ₂	T ₃	SEM	P value
Haemoglobin (g/dl)	11.63	11.59	11.15	0.275	0.395
PCV (%)	34.13	34.29	32.88	0.760	0.368
Glucose (mg/dl)	59.97	61.36	60.08	1.752	0.825
Total protein (g/dl)	6.56	6.64	6.57	0.094	0.805
Albumin (g/dl)	3.01	3.09	3.03	0.037	0.285
Globulin (g/dl)	3.55	3.55	3.54	0.088	0.988
A:G ratio	0.85	0.87	0.86	0.025	0.803
LDH (IU/l)	1149.7	1084.4	1087.4	42.66	0.483
SGPT (IU/l)	27.26	26.16	27.77	1.585	0.764
SGOT (IU/l)	102.2	100.5	102.6	3.060	0.879
Triglycerides (mg/dl)	8.22	7.86	7.44	0.452	0.489
Cholesterol (mg/dl)	83.74	86.99	84.50	3.433	0.784
Urea (mg/dl)	31.06 ^b	27.81 ^a	26.63 ^a	1.004	0.012

^{ab}Means with different superscript in a row differ significantly. T₁, control; T₂, AjO @ 1 ml/calf/day; T₃, AjO @ 2 ml/calf/day; PCV, packed cell volume; LDH, lactate dehydrogenase; SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxalate transaminase.

concentration. Thus these results propose that EO feeding does not influence the function of organs associated with blood metabolites tested in this experiment, except serum urea concentration. Although there was 8.5% inhibition in methane production (ml/kg DDMI), 16.4% higher body weight gain accompanied with 7.74% higher DM intake resulting in 5.61% better feed conversion efficiency in the animals of T₃ group fed 2 ml of essential oil/head/day but statistically it could not reach to significant.

The results indicated that though AjO exhibited *in vitro* antimethanogenic activity but was not able to reduce methane production by feeding to buffalo calves at the level of 2 ml/head/d. There was also no impact on growth performance of the animals, however, AjO worked as protein metabolism modulator which resulted in improved protein utilization efficiency.

ACKNOWLEDGEMENT

The financial assistance provided to senior author in the form of Senior Research Fellowship and research grant provided by ICAR to National Professional Chair are gratefully acknowledged.

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